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Title: Multiple-Resistant Italian ryegrass (*Lolium perenne* spp. *multiflorum*) Populations in Oregon

Abstract approved:

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Italian ryegrass (*Lolium perenne* spp. *multiflorum*) is a common weed management problem in turfgrasses, cereals and non-crop areas in the United States. In Oregon, the number of populations with multiple-resistance continues to increase. To manage these resistant populations, the resistance patterns must be determined. In this study, five Italian ryegrass populations (CT, R1, R2, R3 and R4) from two cropping systems were studied for resistance patterns and mechanisms. The CT population is from a Christmas tree plantation and was resistant to at least six herbicides with four different mechanisms of action: atrazine, diuron (2.4-fold), glyphosate (7.4-fold), hexazinone (3.1-fold), imazapyr (1.8-fold), and sulfometuron. The resistant indices (RI) for sulfometuron and atrazine could not be calculated because 50% growth reduction for the CT population was not reached even with the highest rates applied, 17.6 kg ai $ha^{-1}$and 16 kg ai ha^{-1}, respectively, which are 16 times the recommended field application rates for this two

herbicides. The CT population accumulated less shikimate than the S population. There were two mutations in the CT population, Trp591 to Leu in the ALS gene and Ser264 to Gly in the *psb*A gene, which explain the ALS and PII cross resistance, respectively. R1, R2, R3 and R4 were collected from annual cropping systems. All four populations were resistant to flufenacet. RIs for two populations, R2 and R4, were 8.4 and 5.9, respectively. R2 and R4 also were resistant to mesosulfuron-methyl, pinoxaden, quizalofop and clethodim. R4 was resistant to diuron, but R2 was not. An Asp-2078-Gly substitution in the ACCase gene was found in both R2 and R4 populations, while another Ile-2041-Asn substitution in the ACCase gene was found in the R4 population. These mutations explain the ACCase cross resistance in the R2 and R4 populations. The mechanisms for the glyphosate resistance in the CT population and the flufenacet resistance in R1, R2, R3 and R4 populations were not identified in this study. None of the five populations were resistant to the herbicide pyroxasulfone.

Multiple-Resistant Italian Ryegrass (*Lolium perenne* spp.*multiflorum*) Populations in Oregon

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Mingyang Liu, Author

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CONTRIBUTION OF AUTHORS

Associate Professor Andrew G. Hulting was involved with the research design and writing of Chapter 2 and Chapter 3 of this thesis.

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CHAPTER 1: INTRODUCTION

ITALIAN RYEGRASS

Italian ryegrass (*Lolium perenne* ssp. *multiflorum*), also called annual ryegrass, is an upright annual grass that may also behave like a biennial or short-lived perennial which grows vigorously in winter and early spring. Italian ryegrass is found in roadsides, ditches and crop fields. Italian ryegrass and a related species, perennial ryegrass (*Lolium perenne L.*), can cross and produce offspring which are difficult to identify as either species (Justice et al. 1994). Ryegrasses are cultivated for turf and forage, and sometimes, Italian ryegrass is grown as a cover crop (Kondo et al. 1992).

Italian ryegrass is also known as one of the most problematic weeds in farmland throughout U.S. In Oregon, Italian ryegrass is a competitive weed in winter wheat. Italian ryegrass reduces wheat yield by competition during wheat tillering and interference during wheat harvesting because this weed has a similar growth habit with wheat (Justice et al. 1994). Appleby et al. (1976) reported the yield of wheat could be reduced by 4.2% for each 10 Italian ryegrass plants per m^2. However, some cultural practices such as manipulating wheat seeding density may reduce Italian ryegrass interference with wheat (Appleby and Brewster 1992). Italian ryegrass is also a weed management problem in orchard plantations (Perez and Kogan 2002).

Furthermore, Italian ryegrass is an especially important problem because of the widespread presence of herbicide-resistance populations. Diploid Italian ryegrass is

self-incompatible obligate out-crossing species which allows the resistance genes to spread easily via pollen. Resistant Italian ryegrass populations evloved rapidly because of repeated herbicide use. Italian ryegrass populations resistant to herbicide mechanism of action (MOA) Groups 1, 2, 5, 9, 10 and 15 have been reported in the PNW and other areas of North America (Rauch et al. 2010, Avila and Mallory-Smith 2011, Jasieniuk et al. 2008).

HERBICIDE RESISTANCE

Herbicide resistance is the ability of a weed population to survive a herbicide treatment that previously was known to control the population (WSSA 2012). Herbicide resistance may be the result of natural selection by repeated herbicide applications or gene flow from resistant crops (Mallory-Smith and Sanchez-Olguin 2011). Under normal growing situations, resistant individuals or biotypes remain at very low frequencies in the whole weed population (Mallory-Smith and Sanchez-Olguin 2011, Roux and Reboud 2007). Continually applying a herbicide with the same mode of action (MOA) may result in selection of resistant biotypes which then replace the original population (Roux and Reboud 2007).

ALS-resistance

Acetolactate synthase (ALS) herbicides can be organized into five chemical classes: sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinylthiobenzoate (PTB) and sulfonylaminocarbonyltriazolinone (SCT). ALS is the first enzyme common to the biosynthesis of the branched-chain amino acids valine,

leucine, and isoleucine (Devine and Preston 2000). As reviewed by Yu et al. (2013), ALS resistance can be conferred by reduction in target site sensitivity, ALS overexpression or enhanced rates of ALS herbicide metabolism. In most cases, ALS resistance is due to target site mutations.

ACCase-resistance

Acetyl coenzyme A carboxylase (ACCase) is involved in the first step of lipid synthesis (Devine and Preston 2000). Thus, ACCase inhibitors affect cell membrane production in the meristems of grass plants. ACCase herbicides include three chemical groups: aryloxyphenoxypropionate (APP), cyclohexanedione (CHD), and phenylpyrazolin (PPZ). As reviewed by Beckie and Tardif (2012), ACCase resistance can be conferred by target mutations or enhanced rates of ACCase herbicide metabolism. For target site based ACCase resistance, 11 mutations have been found in seven grass populations.

PSII-resistance

PSII inhibitors include four chemical classes: triazine, triazinone, uracil and urea herbicides. PSII inhibitors reduce electron flow from water to $NADPH^{2+}$ during photosynthesis by binding to the Q_B site on the D1 protein, and preventing quinone from binding to this site (Takano et al. 2008, Perry et al. 2012, Ventrellar and Agostiano 2010). A range of mutations within the QB binding site of the D1 protein may provide resistance to PSII herbicides (Devine and Preston 2000). Furthermore, PSII-resistance due to enhanced rates of herbicide metabolism has been reported (Beckie and Tardif 2012).

EPSPS-resistance

5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an enzyme in the shikimate pathway, in which shikimate-3-phosphate (S3P) reacts with phosphoenolpyruvate to form 5-enolpyruvyl-shikimate-3-phosphate (ESP) (Siehl 1997). Glyphosate can block the shikimate pathway by occupying the binding site of the phosphoenolpyruvate (Siehl 1997). The blockage of the shikimate pathway causes shikimate acid accumulation, which can be used to detect glyphosate activity. EPSPS-resistance can be conferred by target-site mutation, amplification of the EPSPS gene, increased EPSPS activity, and reduced translocation (Cruz-Hipolito et al. 2009, Dickson et al. 2011, Nadual et al. 2008, Preston et al. 2009, Powles and Preston 2006, Perez-Jones et al. 2005, Salas et al. 2012, Shaner 2009).

Cross-resistance

Cross-resistance is resistance to two or more herbicides with the same MOA, and may be due to target site or non-target site resistance (Beckie and Tardif 2012). Non-target site resistance may include enhanced rates of herbicide metabolism or reduced herbicide translocation.

Multiple-resistance

Multiple-resistant weed biotypes may have two to many distinct resistance mechanisms and may exhibit resistance to a few or many herbicide classes (WSSA 2013). In some cases a number of resistance mechanisms, involving both target site and non-target site resistance mechanisms, are present within the same individual (Christopher et

al., 1992). Multiple resistant weed populations are the most complicated to manage. Multiple-resistance is result of either natural selection or accumulation of herbicide resistant genes.

RESEARCH OBJECTIVES

In Oregon, herbicides are widely used to control Italian ryegrass in many cropping systems. Flufenacet, metolachlor, dimethenamid-*p*, acetochlor, diuron, glyphosate, mesosulfuron-methyl, pinoxaden, quizalofop and clethodim are effective herbicides for Italian ryegrass control (Peachey et al. 2012). On the other hand, resistant Italian ryegrass populations evolved rapidly because of repeated herbicide use. Cross-resistance or multiple-resistance Italian ryegrass populations have been reported in the PNW and other areas of North America, which involve several herbicide groups (Rauch et al. 2010, Avila and Mallory-Smith 2011, Jasieniuk et al. 2008). Because the resistance genes can move via pollen and seeds, the spread of multiple-resistant Italian ryegrass is likely to occur and make the management of these populations more difficult.

The objectives of this research were to: 1), identify the resistance patterns and mechanisms in Italian ryegrass populations; and 2), evaluate possible alternative post-emergence and pre-emergence herbicides for management of multiple-resistant Italian ryegrass populations.

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CHAPTER 2: MULTIPLE-RESISTANT ITALIAN RYEGRASS (*LOLIUM* SPP *PERENNE MULTIFLORUM*) POPULATION FROM A CHRISTMAS TREE PLANTATION

ABSTRACT

Multiple-resistance in *Lolium perenne* spp. *multiflorum* has evolved in many areas worldwide. In Oregon, the number of populations with multiple-resistance is increasing. To manage the resistant populations, the resistance patterns must be determined. In this study, a population (CT) suspected to be resistant to sulfometuron and hexazinone, was collected from a Christmas tree plantation. The CT population is resistant to at least six herbicides with four different mechanisms of action: atrazine, diuron (2.4-fold), glyphosate (7.4-fold), hexazinone (3.1-fold), imazapyr (1.8-fold), and sulfometuron. The resistance indices for sulfometuron and atrazine could not be calculated because 50% growth reduction for the CT population was not reached even with the highest rates applied, 17.6 kg ai $ha^{-1}$and 16 kg ai ha^{-1}, respectively, which are 16 times the recommended field application rates of these two herbicides. Acetolactate synthase (ALS) sequencing in the CT population identified a Trp591 to Leu mutation which previously has been reported to provide high level ALS resistance. A Ser264 to Gly mutation was identified in the *psb*A gene which has been reported to impart photosystem II resistance. No previously reported mutation in the 5-enolpyruvylshikimate-3-phosphate synthase gene was found in the CT population. Therefore the mechanism of glyphosate resistance was not identified for the CT population. However, there was less shikimatic acid accumulation in the CT biotype than in the susceptible biotype after treatment with

glyphosate at 0.6 kg ai ha^{-1}. In Oregon, glufosinate, clethodim, sethoxydim, and fluazifop are registered for use in Christmas tree production and could be used to manage this Italian ryegrass population.

Key Words: herbicide resistance, dose-response studies.

INTRODUCTION

Italian ryegrass is a weed problem in many different cropping systems of the Pacific Northwest (PNW) (Hulting et al. 2012, Jasieniuk et al. 2008, Appleby and Brewster 1992). Resistant Italian ryegrass populations evolved rapidly because of repeated herbicide use in these cropping systems. Italian ryegrass populations resistant to MOA Groups 1, 2, 5, 9, 10 and 15 have been reported in the PNW and other areas of North America (Rauch et al. 2010, Avila and Mallory-Smith 2011, Jasieniuk et al. 2008). Some of these populations exhibit cross-resistance and/or multiple-resistance involving several herbicide groups.

Since the first acetolactate synthase (ALS) inhibitor resistance was reported in prickly lettuce (*Lactuca serriola*) in 1987 (Mallory-Smith et al. 1990), ALS resistant weed biotypes have been widely reported (Kuk et al. 2007, Kuk et al. 2008, Chandi et al. 2011). The prickly lettuce population evolved within 5 years of the first use of sulfonylurea herbicide in the same field (Mallory-Smith et al. 1990). ALS resistance is one of the most commonly evolved resistances (Beckie et al. 2006). By 2013, 129 populations of ALS resistant weed were reported in 32 countries (International Survey of

Herbicide Resistant Weeds, 2013). Target-site mutations may confer ALS herbicide resistance at different levels to the five ALS inhibiting chemical groups, sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinylthiobenzoate (PTB) and sulfonylaminocarbonyltriazolinone (SCT). In field-evolved ALS resistant weed populations, 24 substitutions have been reported at eight amino acid positions in the ALS gene (Han et al. 2012). Eight resistant *Lolium rigidum Gaudin* or *Lolium* spp. populations have been reported with six substitutions at two positions (Pro-97-Ala, Pro-97-Arg, Pro-97-Leu, Pro-97-Ser, Pro-97-Thr or Trp-574-Leu) (Beckie et al. 2012).

Acetyl-coenzyme A carboxylase (ACCase) inhibitors include the aryloxyphenoxy propionate (APP), phenylpyrazolin (pinoxaden) and cyclohexanedione (CHD) chemical families. ACCase herbicides inhibit the chloroplastic ACCase in Gramineae and prevent fatty acid synthesis, thus affecting cell membrane production in the meristems of grass plants (Delye et al. 2002). Similar to ALS resistance, ACCase herbicide resistance evolved quickly (Beckie and Tardif 2012). By 2013, 42 ACCase resistant populations were reported in 28 countries (International Survey of Herbicide Resistant Weeds 2013). ACCase resistance has been conferred by 11 mutations at seven positions with seven of them found in ryegrass populations (*Lolium* spp.) (Ile-1781-Leu, Trp-1999-Cys, Trp-2027- Cys, Ile-2041-Asn, Ile-2041-Val, Asp-2078-Gly, Cys-2088-Arg) (Beckie and Tardif 2012).

Group 5 and Group 7 herbicides are photosystem II (PSII) inhibitors. PSII inhibitors include the chemical classes of trazine, trazinone, uracils and urea herbicides. They provide selective residual control of several broadleaf weeds and certain grass species in

many crop and non-crop environments (Devine and Preston 2000). Herbicides in Group 5 and Group 7 have the same target-site but different binding behaviors. PSII inhibitors kill plants by binding to the protein D1, thereby preventing the flow of free electrons generated in PSII (Takano et al. 2008, Perry et al. 2012, Ventrellar and Agostiano 2010). Mutations occurring in the *psb*A gene, which encodes the D1 protein, may result in PSII herbicide resistance (Devine and Preston 2000). At least five substitutions that confer resistance have been reported in the *psb*A gene (Val-219-Ile, Ser-264-Gly, Ser-264-Thr, Asn-266-Thr, Phe-255-Ile and Ala-251-Val) (Beckie and Tardif 2012).

Glyphosate affects plants by blocking the shikimate pathway (Harring et al. 1998, Dev et al. 2012). Glyphosate inhibits the enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate to form 5-enolpyruvyl-shikimate-3-phosphate (ESP) (Siehl 1997). There are several glyphosate resistance mechanisms reported. These mechanisms include target-site mutation, amplification of the EPSPS gene, increased EPSPS activity, and reduced translocation (Cruz-Hipolito et al. 2009, Dickson et al. 2011, Nadual et al. 2008, Preston et al. 2009, Powles and Preston 2006, Perez-Jones et al. 2005, Salas et al. 2012, Shaner 2009). For target-site based resistance, an amino acid substitution at Pro106 of EPSPS is important because it most likely regulates the glyphosate binding site (Healy-Fried et al. 2007). This mechanism is known to contribute glyphosate resistance in several different species (Preston et al. 2009).

Glyphosate is a systemic herbicide and can be absorbed by the plant, translocated to actively growing tissues and inhibits aromatic amino acid synthesis (Shaner et al. 2012).

At low application rates, reduced translocation mechanisms may block the translocation of glyphosate to protect the whole plant (Shaner 2009, Shaner et al. 2012). Compared with susceptible plants, resistant plants with this mechanism of resistance have lower glyphosate translocation rates following glyphosate application (Nadual et al. 2008, Perez-Jones et al. 2007, Wakelin et al. 2004). As indicated by Shaner (2009), several potential mechanisms may block the glyphosate from or pump the glyphosate away from the target site when glyphosate is taken up by plant cells. However, the mechanism of reduced glyphosate translocation is still unclear.

Another mechanism involved in glyphosate resistance is EPSPS overexpression. This mechanism may include both EPSPS gene amplification and increased enzyme activity (Pline-Srnic 2004, Shaner et al. 2012). Unlike the target mutation mechanism, the EPSPS enzyme in the resistant plants remains in the sensitive form (Pline-Srnic 2004, Salas et al. 2011). The increased EPSPS gene copies or enzyme activity enhances the glyphosate resistance. Some resistant biotypes may have both forms of EPSPS overexpression.

Multiple resistant biotypes have more than one resistance mechanism. This kind of resistance makes weed management difficult because of limited chemical control options in various crops. Multiple-resistance can occur via field selection by herbicides with different sites-of-action or through accumulation of resistance genes as a result of pollination between related species (Beckie and Tardif 2012, Mallory-Smith and Sanchez-Olguin 2010, Rauch et al. 2010). The objectives of this study were to identify

the resistance pattern and evaluate the resistance levels of an Italian ryegrass population from a Christmas tree plantation in Oregon.

METHODS AND MATERIALS

Plant materials

Seeds from an Italian ryegrass population (CT) were collected from a Christmas tree farm near Molalla, OR, in 2010. The Italian ryegrass population survived two field applications of premixed hexazinone and sulfometuron. Both hexazinone and sulfometuron had been used previously at this site. A known herbicide-susceptible population (S) was used as control in this study.

General greenhouse study procedures

Greenhouse studies were conducted with 25/20 C day/night temperatures with ambient sunlight plus grow lights providing 14 hr light above 25 mW cm^{-2} per day. Herbicide treatments were applied using an experimental spray chamber equipped with 8004 nozzles calibrated to deliver 187 L ha^{-1} at 276 Kpa.

For pre-emergence herbicide treatments, one experimental unit was a 25 by 50 by 6 cm plastic tray. Each tray was filled with commercial potting media (Sunshine Mix 1 Potting Mix, Sun Gro Horticulture, Inc., 110th Ave. NE, Suite 490, Bellevue, WA 98004) and divided into two equal parts. Fifty seeds of S and 50 seeds of CT, respectively, were planted in each tray. Herbicides were applied one day after seeding. Three replications were used per rate for each herbicide, and the study was repeated.

For post-emergence herbicide treatments, seeds from both populations were placed in 9 cm petri dishes containing moistened blotter paper for germination. When the coleoptiles reached 1.5 cm, the seedlings were transplanted to a 267-ml plastic pot containing commercial potting mix. Six pots of S seedlings and six pots of CT seedlings were considered to be one experimental unit. Plants were grown under greenhouse conditions for about one week before herbicides were applied. Herbicide treatments were applied at the 2-3 leaf growth stage. Three replications (total of 18 pots) were used per rate for each herbicide, and the study was repeated.

Herbicide Resistance Screening Assays

The CT and S population were screened with herbicides recommended to control Italian ryegrass in Christmas tree production systems (Peachey et al. 2012) (Table 2.1). The recommended field rate and half of the recommended field rate were used for each herbicide. Herbicide efficacy was estimated visually at 21 d (PRE-) or 14 d (POST-) after herbicide applications. For POST- herbicides, the injury of the plants was rated on a scale from 0 (no injury) to 10 (death). For PRE- herbicide studies, the experimental units with the same germination as the control group were rated as 0, and the units with no emerged seedlings were rated as 10. The Student t-test using R software (R statistical software, R development team, http: //www.r-project.org/.) was used to analyze these visual rating data.

Dose-Response Study

Those herbicides (atrazine, diuron, glyphosate, hexazinone, imazapyr, sulfometuron), to which the CT population survived the recommended rate, were included in the dose-response studies. Seven rates including the control were used in this study (Table 2.2). The experimental design was a completely randomized block with 3 replications, and the study was repeated.

Seedlings were cut at the soil surface at 14 d (POST-) or 21 d (PRE-) and dried at 60 C for 72 h. The percent dry weight of the treated plants relative to the untreated control group was calculated. R and Levene's ANOVA were used to compare differences between treatments. The homogeneity of variances between populations with the same treatments was compared. GR_{50} values were estimated using the drc package in R (DRC package version 2.1-4).

Shikimic Acid Assay

The blockage of the shikimate pathway causes the accumulation of shikimic acid in fresh leaf tissue in glyphosate susceptible plants (Nol et al. 2012). Compared with visual inspection of whole plant injury, measuring accumulation of shikimic acid is a rapid and accurate quantitative technique for screening glyphosate efficacy and resistance (Harring et al. 1998, Nol et al. 2012, Ribeiro et al. 2008). A high accumulation of shikimic acid indicates that glyphosate reached the target site and inhibited the shikimate pathway. In contrast, lower than normal shikimic acid accumulation may indicate glyphosate tolerance or resistance.

Seeds from CT and S populations were germinated in 9 cm petri dishes containing moistened blotter paper and set in a growth chamber. When the coleoptiles reached 1.5 cm, seedlings from the CT and S populations were transplanted to 267-ml plastic pots containing commercial potting mix. Plants were grown under the same greenhouse conditions described previously. When the plants reached on average the 2-3 leaf stage, glyphosate was applied at 600 g ae ha^{-1}. At 24, 48, 72, 96 and 120 h after treatment (HAT), tissue was collected from six treated and six untreated plants in each population. Shikimate acid extraction was performed according to Perez-Jones et al. (2007). Briefly, leaves were harvested, chopped and 0.10 g fresh tissue was ground in liquid nitrogen. After the liquid nitrogen evaporated, 1 mL of 0.25 N HCl was added, and the solution vortexed. The samples were incubated at 37 C in a water bath for 45 min and centrifuged for 10 min at 13,000 rpm. Twenty-five milliliter aliquots from each sample were pipetted into a 96 well plate with each well containing 100 µL mixture of 0.25% periodic acid and 0.25% sodium meta periodate to oxidize the shikimic acid. The samples were then incubated 30 min at 37 C. After incubation, samples were mixed in each well with 100 µl mixture of 0.6 N NaOH and 0.22 M Na_2SO_3 and optical density measured at 380 nm by a spectrophotometer (Molecular Device, Sunnyvale, CA 94089). A standard curve was calculated by measuring shikimic acid concentration at 0.0001, 0.001 and 0.01 µg ml^{-1}, in order to determine shikimic acid concentration from the optical data. The difference of the shikimic acid concentrations between treated and untreated plants in each population was calculated as shikimic acid accumulation.

Gene Sequencing

Seedlings of the CT population surviving the highest application rate of glyphosate, sulfometuron, and hexazinone in the dose-response studies were selected for gene sequencing. Fresh plant tissue was harvested independently from 4 healthy plants in each treatment group and from 2 untreated plants in the S population. Total cellular DNA was extracted immediately from harvested fresh tissue using a DNeasy Plant Mini Kit (Qiagen) following the mini protocol (DNeasy plant handbook). Polymerase chain reactions (PCRs) were performed to amplify the regions of interest. The reaction mixture contained 1.15 µl DNA solution, 0.2 µM dntp, 0.5 µM forward primer, 0.5 µM reverse primer, 1 µl 10× PCR buffer, 0.06 unit *Tag* DNA polymerase and 6.74 µl water. The primers and PCR thermal cycler varied depending on the regions of interest (Table 2.3). The PCR products were purified with TOPO TA Cloning Kit for Sequencing following the manufacturer's instructions (TOPO TA Cloning Kit for Sequencing, Invitorgen). Cloning products were purified using a QIAquick PCR Purification Kit following the vacuum protocol in QIAquick Spin Handbook. PCR products were sent to the Center for Genome Research and Biocomputing (CGRB), Oregon State University, for sequencing. Ten clones per PCR product were loaded on an automated ABI PRISM 3770 sequencer after treatment with a BigDye Terminator v.3.1 cycle sequencing kit. Each clone was sequenced in both directions. Sequencing results were edited with Finch TV and aligned using CLC Sequence Viewer v.6.1. Sequences obtained from GenBank were used for reference.

RESULTS

Herbicide Resistance Screening Assays

The CT population was resistant to six herbicides in four different groups used in this study (Table 2.1). The population was resistant to ALS inhibitors (Group 2), PSII inhibitors (Group 5 and 7) and the EPSPS inhibitor (Group 9). The CT population was not resistant to clethodim, sethoxydim and fluazifop in Group 1, glufosinate in Group 10, or flufenacet and pyroxasulfone in Group 15.

Dose-Response Study

The S population was controlled by all herbicides used in the dose-response study at the recommended rate. The resistant indexes (RI) were 2.46, 7.40, 3.10 and 1.78 for diuron, glyphosate, hexazinone and imazapyr, respectively, compared with the S population (Table 2.4). The herbicide effects were positively related to the doses of the herbicides. Growth of plants from the CT population treated with atrazine and sulfometuron did not reach 50% reduction at 16,000 and 17,600 g ai ha^{-1}, respectively, which are 16 times the recommended field application rate. Therefore, a resistant index could not be calculated for atrazine or sulfometuron.

Shikimic Acid Assays

At 24 HAT with glyphosate at 600 g ae ha^{-1}, accumulated shikimic acid was detected in both CT and S populations (Figure 2.1). The shikimic acid concentration increased in both populations at 48, 72, 96 and 120 HAT. However, plants from the S population had higher shikimic acid concentration than plants from the CT population at all extraction times. At the end of this assay, 120 HAT, the shikimic acid accumulation in both

populations was greater than the untreated plants. At 120 HAT, most of the plants from the S population were chlorotic and necrotic while resistant plants in CT group were not.

Gene Sequencing

There was no mutation at site 106 or surrounding positions of the EPSPS gene in either the S or CT populations. In this study, we did not determine the numbers of copies of EPSPS genes, which is one known mechanism of the glyphosate resistance.

Comparison of the PCR fragments of the ALS gene from CT and S populations revealed a single nucleotide change of TGG to TTG in the CT population (Table 2.5). This change leads to a Trp-591-Leu substitution. As reported in previous studies, this mutation contributes to resistance to all classes of ALS inhibitor herbicides (Devine and Preston 2005, Bernasconi et al. 1995).

A single nucleotide change of AGT to GGT, leading to a Ser-264-Gly substitution, was found in *psb*A gene of the CT population (Table 2.6). This mutation has been reported in most s-triazine resistance cases (Devine and Preston 2000), and causes decreased binding of s-triazine herbicides to the D1 protein (Perry et al. 2012, Jia et al. 2007).

DISCUSSION

Multiple-herbicide resistance was identified in the CT population. The CT population was not resistant to glufosinate, clethodim, sethoxydim, fluazifop or flufenacet which can be used as alternative controls for the CT population. All of these herbicides are

registered for use in Christmas trees in Oregon. Furthermore, the CT population was not resistant to pyroxasulfone.

The Trp-591-Leu mutation found in the ALS gene can explain the Group 2 cross-resistance of the CT population. Trp-591-Leu has been reported in at least 26 species including *Lolium* spp. and *Lolium rigidum Gaudin*, to confer the resistance to the five chemical groups of ALS inhibitors (Beckie and Tardif 2012). As a result, we expect the CT population to be resistant to other Group 2 herbicides in addition to imazapyr and sulfometuron which were evaluated in this study.

The Ser-264-Gly is the most common mutation found in field-selected PSII resistant plants and was found in the CT population (Perez-Jones et al. 2009). The CT population was resistant to atrazine at a high level and was resistant to hexazinone and diuron with RIs of 3.10 and 2.46, respectively. In previous studies, three possible mutations at Ser264, Ser-264-Gly, Ser-264-Ala or Ser-264-Thr, conferred high level triazine (atrazine) resistance, but only Ser-264-Gly provides triazinone (hexazinone) resistance (Devine and Preston 2000, Perez-Jones et al. 2009, Beckie and Tardif 2012). Though many studies indicate that Ser-264-Gly may not confer urea (diuron) resistance (Devine and Preston 2000, Perez-Jones et al. 2009), low level urea resistance was reported in some weeds with this mutation (Masabni and Zandstra 1999). In the CT population, we did not identify any other mutations in *psb*A gene. Therefore, it appears that the diuron resistance was likely due to the Ser-264-Gly mutation.

Both shikimate and whole plant dose-response studies confirmed glyphosate resistance (7.4 fold). No reported mutation was found in EPSPS gene, which indicates the

resistance is not likely target based. If the glyphosate resistance is based on reduced translocation, the shikimate acid accumulation should not be observed when the application rate is low and increases when glyphosate application rate is high (Perez-Jones et al. 2007, Nadual et al. 2008, Wakelin et al. 2004). If the glyphosate rate is high enough, the protective mechanism will lose its effectiveness and glyphosate will reach the target site (Shaner 2009). The capability of this protection varies with the resistance levels. In the shikimate test, shikimate acid accumulated in the CT population after treatment with glyphosate at 600 g ae ha^{-1}. The CT population was resistant to glyphosate with a GR_{50} of 349 g ae ha^{-1} which was 7.4 fold greater than the S population. Though a lower application rate was not tested in this study, compared with previous studies, we might expect a lower accumulation level if this resistance is only based on reduced translocation. Therefore, reduced translocation may not be the only reason for resistance in the CT population. Further studies need to be conducted to identify the mechanism of glyphosate resistance in this population.

In the CT population, herbicide resistance could have evolved from long term herbicide applications or from gene flow from other resistant populations. Because sulfometuron and hexazinone have been used on this farm, the target site mutation based ALS and PSII resistance was most likely selected by the repeated herbicide use. Glyphosate was also used in this area for weed management, therefore the EPSPS resistance was most likely selected by the repeated glyphosate use. In contrast to the EPSPS gene and ALS gene, *psb*A is maternally inherited (Perez-Jones et al. 2009). Therefore, the PSII resistance will be less likely to be spread. Furthermore, PSII

resistance may cause fitness penalty in the plants, this population may less competitively than other populations.

Before an herbicide is applied, herbicide resistant weed biotypes may be present in low frequencies in the wild population (Boddy et al. 2012, Mallory-Smith and Sanchez Olguin 2010). Though the progeny fitness or competitive ability was not tested in this study, we did notice that the dry weight of the CT population was less than the S population (data not shown). Because the S population was not from the same site, it will be necessary to identify susceptible plants in the CT population and produce seeds before fitness studies can be conducted using this population.

Although this population has multiple resistant mechanisms, there are still chemical options for its control. These options include several recommended herbicides in Group 1 and Group 15. Pyroxasulfone could also be an optional option for its control, if and when it is labeled for use in Christmas tree production. However, this population could be a new source of resistance to other Italian ryegrass populations through seed and pollen movement.

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Table 2.1. Injury levels (0-10)[a] of treated Italian ryegrass population compared to the untreated control.

Herbicide[b]	Group	Timing	Rate[c]	Populations[d]		P[e]
				CT		
clethodim	1	POST	136	8.50	8.67	0.60
		POST	68	8.33	8.33	1.00
sethoxydim	1	POST	270	9.33	9.17	0.72
		POST	135	8.83	9.00	0.69
fluazifop	1	POST	184	9.50	9.33	0.69
		POST	92	8.17 S	8.50	0.58
imazapyr	2	POST	840	5.17	8.33	3.79E-05
		POST	420	4.00	6.67	1.47E-03
sulfometuron	2	POST	1100	1.50	8.17	1.78E-07
		POST	500	0.83	6.50	3.70E-08
hexazinone	5	POST	1340	3.33	8.17	8.82E-07
		POST	670	2.67	6.67	2.21E-05
atrazine	5	POST	1440	2.33	7.83	2.64E-07
		POST	720	2.00	7.33	7.90E-07
diuron	7	PRE	2000	5.67	8.33	2.10E-04
		PRE	1000	5.00	7.50	1.64E-04
glyphosate	9	POST	1000[f]	4.83	9.17	7.63E-05
		POST	500[f]		8.17	3.55E-04
gulfosinate	10	POST	1000	9.33	9.67	0.29
		POST	500	8.00	8.50	0.27
flufenacet	15	PRE	380	8.33	8.33	1.00
		PRE	190	7.83	7.50	0.54
pyroxasulfone	15	PRE	120	10.00	10.00	N/A
		PRE	60	10.00	10.00	N/A

5.17

[a] For POST- herbicides the injury of the plants was rated on a scale from 0 (no injury) to 10 (death). For PRE- herbicide studies, the experimental units with the same germination as the control group were rated as 0, and the units with no emerged seedlings were rated as 10.

[b] clethodim (SelectMax, 12.6% by weight clethodim EC)
sethoxydim (Poast, 18% by weight sethoxydim EC)
fluazifop (Fusilade Forte, 128g L^{-1} EC)
imazapyr (Arsenal, 53.1% by weight isopropylamine salt of imazapyr EC)
sulfometuron (Oust, 75% by weight sulfometuron methyl XP)
hexazinone (Velpar, 75% by weight hexazinone DF)
atrazine (Atranex, 90% by weight atrazine WG)
glyphosate (Roundup Power Max, 540 g L-1 glyphosate EC)
glufosinate (Rely 200, 18.19% by weight glufosinate ammonium EC)
flufenacet (Define 60% flufenacet by weight DF)
diuron (Karmex, 80% by weight diuron DF)
pyroxasulfone (Experimental code: KIH465, 85% pyroxasulfone by weight WP)
[c] X and 1/2 X of recommend use rate
[d] Data pooled with 6 replications.
[e] P value of hypothesis that there is no difference between CT and S populations.
[f] g ae ha^{-1}

Table 2.2. Herbicides and rates used in the dose-response study.

Herbicide	Populations	Rate (g ai ha^{-1})								
		0.0625x	0.125x	0.25x	0.5x	1x [a]	2x	4x	8x	16x
atrazine	CT				500	1000	2000	4000	8000	16000
	S		125	250	500	1000	2000	4000		
diuron	CT	125	250	500	1000	2000	4000			
	S	125	250	500	1000	2000	4000			
glyphosate[b]	CT			250	500	1000	2000	4000	8000	
	S	62.5	125	250	500	1000	2000			
hexazinone	CT				670	1340	2680	5360	10720	21440
	S		167.5	335	670	1340	2680	5360		
imazapyr	CT				420	840	1680	3360	6720	13440
	S		105	210	420	840	1680	3360		
sulfometuron	CT				550	1100	2200	4400	8800	17600
	S		137.5	275	550	1100	2200	4400		

[a] 1X= recommended rate.

[b] g ae ha^{-1}

Table 2.3. Primers used for sequencing EPSPS, ALS, *psb*A.

Primer[a]	Gene	Sequence 5' to 3'
EPSPS F[b]	EPSPS	AGCTGTAGTCGTTGGCTGTG
EPSPS R	EPSPS	TCGCTCCCTCATTCTTGGTA
ALS1 F	ALS	ATCACCAACCACCTCTTCC
ALS1 R	ALS	ATCTGCTGCTGGATGTCCTT
ALS2 F	ALS	TGGGCGGCTCAGTATTACA
ALS2 R	ALS	ATAGGCAGCACATGCTCCTG
*psb*A F	*psb*A	GGATGGTTTGGTGTTTTG
*psb*A R	*psb*A	TAGAGGGAAGTTGTGAGC

[a] EPSPS designed based on *Lolium multiflorum* (DQ153168.2) and *Lolium rigidum* (DQ303404.1 and EU350208.1)
ALS designed based on *Lolium rigidum* (EF411170.1 and EF411171.1)
PSII designed by based on *Lolium multiflorum* (EU360732.1 and EL664115.1)
[b] F: forward; R: Reverse

Table 2.4. Regression equations for the dose-response study[a].

Herbicide	Populations	Regression equation	R^2	GR_{50} g ai ha^{-1}	SE	R/S
atrazine	CT	NC[b]	NC	>16000[c]		NC
	S	y=14.74+88.07/(1+exp(1.78(log(x)-2.59)))	0.98	386.41	3.60	-
diuron	CT	y=-2.57+104.51/(1+exp(1.89(log(x)-2.59)))	0.92	387.67	1.94	2.46
	S	y=-1.61+101.36/(1+exp(1.89(log(x)-2.20)))	0.96	157.42	1.34	-
glyphosate	CT	y=18.13+81.83/(1+exp(1.16(log(x)-2.54)))	0.98	349.41	1.23	7.40
	S	y=14.41+85.63/(1+exp(1.50(log(x)-1.67)))	0.99	47.24	1.08	-
hexazinone	CT	y=13.71+86.56/(1+exp(1.12(log(x)-2.87)))	0.93	734.54	1.77	3.10
	S	y=16.07+83.99/(1+exp(2.76(log(x)-2.37)))	0.99	236.68	1.13	-
imazapyr	CT	y=22.50+77.04/(1+exp(1.33(log(x)-3.12)))	0.85	1313.42	1.35	1.78
	S	y=-7.93+108.97/(1+exp(0.87(log(x)-2.87)))	0.88	738.49	2.01	-
sulfometuron	CT	NC	NC	>17600[c]		NC
	S	y=17.88+82.25/(1+exp(0.83(log(x)-2.31)))	0.97	204.99	0.96	-

[a] Pooled data for 6 replications
[b] Not possible to calculate
[c] Greater than the highest rate

Table 2.5. Comparison of ALS gene sequences from S and CT populations.

Amino acid	M	V	V	Q	W	E	D	R	F	Y	K
Amino acid number	587	588	589	590	591	592	593	594	595	596	597
Nucleotide sequence	ATG	GTG	GTG	CAG	TGG	GAG	GAC	AGG	TTT	TAC	AAA
S	-[a]	-	-	-	TGG	-	-	-	-	-	AAA
CT	-	-	-	-	TTG	-	-	-	-	-	AAG
Amino acid in CT							L[b]				K[c]

[a] – means there was no difference of protein coding nucleotides between the two populations.

[b] Leucine was formed because of the mutation in CT population instead of Aspartic acid.

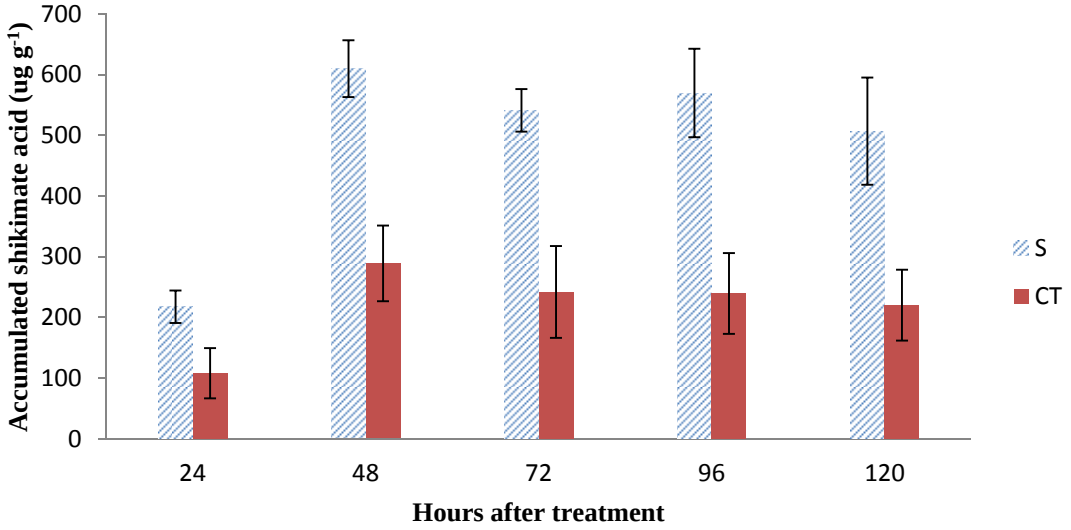
[c] silent mutation

Table 2.6. Comparison of *psb*A gene sequences from S and CT populations.

Amino acid	L	I	F	Q	Y	A	S	F	N	N	S
Amino acid number	258	259	260	261	262	263	264	265	266	267	268
Nucleotide sequence	ATT	AAC	TTC	CAA	TAT	GCT	AGT	TTC	AAC	AAC	TCT
S	-[a]	-	-	-	-	-	AGT	-	-	-	-
CT	-	-	-	-	-	-	GGT	-	-	-	-
Amino acid in CT							G[b]				

[a] – means there was no difference of protein coding nucleotides between the two populations.
[b] Glycine was formed because of the mutation instead of Serine.

Figure 2.1. Shikimate acid accumulation after treatment with glyphosate at 600 g ae ha^{-1}.



CHAPTER 3: MULTIPLE-RESISTANT ITALIAN RYEGRASS (*LOLIUM PERENNE* SSP. *MULTIFLORUM*) POPULATIONS FROM WINTER WHEAT FIELDS IN OREGON

ABSTRACT

Flufenacet is widely used in the Pacific Northwest (PNW) of the U.S. to control Italian ryegrass in winter wheat (*Triticum aestivum L.*) production fields. Recently, four populations of Italian ryegrass in Oregon were identified that survived flufenacet applications under typical winter wheat production conditions. Seed from these populations was collected and greenhouse studies were conducted to confirm and quantify the flufenacet resistance levels of these populations. Resistance patterns were determined to identify alternative herbicides for management. Another objective of this study was to determine the response of the suspected flufenacet resistant Italian ryegrass-populations to pyroxasulfone, a herbicide in the same group, which is expected to be effective for management of Italian ryegrass in various crops. Our results indicate that the four populations were resistant to flufenacet, but were not resistant to pyroxasulfone. The four populations were controlled by pyroxasulfone at rates greater than 7.5 g ai ha^{-1}. Two selected populations, R2 and R4, were resistant to mesosulfuron-methyl, pinoxaden, quizalofop, clethodim, but not to metolachlor, glyphosate, acetochlor and dimethenamid-p. R4 was resistant to diuron, but R2 was not. The estimated flufenacet doses required for 50% growth reduction (GR_{50}) were 438 g ai ha^{-1} (R2) and 308 g ai ha^{-1} (R4). Resistance indices (RI) for the R2 and R4 populations were 8.4 and 5.9, respectively, for flufenacet compared to a susceptible population (S). An Asp-2078-Gly substitution was found in R2 and R4 population, while a Ile-2041-Asn was found only in R4 population. Multiple resistant Italian ryegrass populations in this study were not resistant to other Group 15 herbicides. Group 1 herbicides did not control these populations because of target site mutation based cross-resistance.

Key Words: herbicide resistance, dose-response studies.

INTRODUCTION

The competition between weed and wheat plants for light, water, and nutrients can reduce wheat yield significantly (Liebl and Worsham 1987). Weeds also may inhibit wheat growth and grain yield by releasing allelopathic chemicals which are toxic to wheat plants (Liebl and Worsham 1987, Appleby et al. 1976). In addition, weeds or weed seeds contaminating harvested wheat may reduce grain quality and impact wheat end uses (Crutchfield et al. 1985, Gerhards and Christensen 2003). Therefore, weed management is a very important practice in wheat production.

Italian ryegrass is an annual ryegrass native to temperate Europe, and is commonly found throughout the U.S.A. (Whitson 2006, Avila-Garcia and Mallory-Smith 2011). Because Italian ryegrass has a similar growth habit as winter cereal crops, it is an extremely competitive weed and has become a significant management concern in winter wheat production in the PNW and in the Southeastern U.S. Reductions of winter wheat grain yield were positively related to Italian ryegrass density, and reached 80% in previously reported studies (Liebl and Worsham 1987, Appleby et al. 1976, Appleby and Brewster 1992). The yield loss caused by Italian ryegrass is mainly due to competition for soil nutrients during winter wheat tillering growth stages and the interference during harvest (Liebl and Worsham 1987, Justice et al. 1994, Chandi et al. 2011). In Oregon, flufenacet, metolachlor, dimethenamid-p, acetochlor, diuron, glyphosate, mesosulfuron-methyl, pinoxaden, quizalofop and clethodim are herbicides used to control Italian ryegrass in cereal production systems (Peachey et al. 2012).

Flufenacet is a PRE- or early POST-applied herbicide that controls annual grasses and broadleaf weeds in corn, soybeans and wheat (Anonymous 2007). Flufenacet is applied to the soil surface or incorporated, and is moderately stable in the soil environment

(EPA 1998). Flufenacet inhibits very-long-chain fatty acid biosynthesis (Group 15 mode of action) in plants (Soltania et al. 2005). In the PNW, flufenacet (Define) and its mixture with metribuzin (Axiom) are commonly used for PRE- or early POST- emergence control of Italian ryegrass (Peachey et al. 2012). Flufenacet plus metribuzin controls Italian ryegrass in winter wheat without significant long-term injury to the winter wheat (Grey et al. 2003, Koepke-Hill et al. 2011).

Pyroxasulfone is a recently introduced herbicide developed by Kumiai Chemical Industry Co., Ltd. and Ihara Chemical Industry Co., Ltd (Anonymous 2011). It is in the pyrazole chemical class, and has been classified as a Group 15 mode of action herbicide (EPA 2012, Tanetani et al. 2009). Pyroxasulfone provides PRE control of many grass and broadleaf weeds (Anonymous 2011, Knezevic et al. 2009, King and Garcia 2008). Pyroxasulfone is currently registered for use in corn, and has potential uses in sunflower, soybean, wheat, orchards, vineyard and non-crop areas (EPA 2012, Shikkema et al. 2008). Pyroxasulfone could be an alternative to flufenacet for control of Italian ryegrass populations (Hulting et al. 2012). In other studies, pyroxasulfone was reported to control weeds resistant to glyphosate, trifluralin, cinmethylin and diuron (King and Garcia 2008, Walsh et al. 2011). No resistance has been reported to pyroxasulfone.

Italian ryegrass populations resistant to flufenacet plus metribuzin have been reported (Rauch et al. 2010). According to a herbicide-resistant Italian ryegrass survey conducted in 2006 and 2007, 12% of 75 populations collected from northern Idaho and eastern Washington exhibited varying levels of resistance to flufenacet plus metribuzin. In Oregon, growers also have reported reduced control of Italian ryegrass with flufenacet plus metribuzin. The objectives of this study were to quantify resistance of these

populations to flufenacet, characterize the resistance patterns of the populations and identify possible alternative herbicides to manage these resistant populations.

MATERIALS AND METHODS

Seed collection

Four Italian ryegrass populations suspected of being resistant to flufenacet were evaluated in this study. Three of them (R1, R2 and R3) were collected in 2010 from winter wheat fields in Linn County, OR. These populations were collected within 1.5 km of each other on the same farm from fields in small grain rotations. Population (R4) was collected in 2011 from a winter wheat field in Washington County, OR, approximately 64 km from the other populations, in a field that had a winter wheat-red clover (*Trifolium pratense* L.)-field pea (*Pisum sativum* L.) rotation. The crop rotation history at each location varied, but was representative of the crops grown in typical seed production cropping systems in western OR. All fields had an extensive history of Group 1, 2, 3, 5, 7, 9 and 15 herbicide applications to manage Italian ryegrass populations in both cereal grain and broadleaf crops. An Italian ryegrass population (S), which is known to be herbicide susceptible, was used in this study as a control population.

General greenhouse methods

For each herbicide, experimental units were arranged in a completely randomized design with three replications (total of 18 units), and the experiments were repeated. Herbicides were applied in an experimental spray chamber at an application volume of 187 L ha^{-1} at 276 Kpa. Adjuvants were used as labels required. Plants were grown under ambient light

conditions and supplemented by grow lights to achieve 14 hr light greater than 25 mW cm^{-2} per day. Day/night temperatures were maintained at 25/20 C. A preliminary study was conducted to compare the effect of soil type on flufenacet and pyroxasulfone activity. There was no difference in results whether potting mix or a silt loam soil was used (data not shown). Therefore, a commercial potting mix (Sunshine Mix 1 Potting Mix, Sun Gro Horticulture, Inc., 110th Ave. NE, Suite 490, Bellevue, WA 98004) was used in all studies.

Herbicide screening assays

Flufenacet and pyroxasulfone screening. Italian ryegrass seeds were planted in 25 by 50 by 6 cm plastic trays containing commercial potting mix. Each tray was divided into two equal parts. Fifty seeds of the S and 50 seeds of R1, R2, R3 or R4, respectively, were planted in each tray. The trays were placed in a greenhouse overnight and were then treated with flufenacet or pyroxasulfone. Flufenacet (Define 60% flufenacet by weight DF) was applied at 0, 190 and 380 g ai ha^{-1}, while pyroxasulfone (Experimental code: KIH465, 85% pyroxasulfone by weight WP) was applied at 0, 60 and 120 g ai ha^{-1}.

Pre-emergence alternative herbicide screening. R2, R4 and S were planted using the same methods as described above and placed in a greenhouse overnight. Four pre-emergent (PRE) herbicides including, metolachlor (Dual II Magnum, 916 g L^{-1} s-metolachlor EC), dimethenamid-*p* (Outlook, 720 g L^{-1} dimethenamid-*p* EC), acetochlor (Surpass, 768 g L^{-1} acetochlor EC), diuron (Karmex, 80% by weight diuron DF) were applied at two rates (Table 3.2).

Post-emergence alternative herbicide screening. Italian ryegrass seeds from R2, R4 and S populations were set in petri dishes containing water moistened blotter paper for

germination. When seedlings reached 1.5 cm, they were transplanted to 267-ml plastic pots containing commercial potting mix. Each experimental unit contained 12 pots, 6 pots for R2 or R4 and 6 for S. Five post-emergent (POST) herbicides including, glyphosate (Roundup Power Max, 540 g L^{-1} glyphosate EC), mesosulfuron-methyl (Osprey, 4.5% by weight mesosulfuron-methyl WDG), pinoxaden (Axial, 50 g L^{-1} pinoxaden XL), quizalofop (Assure II, 10.3% by weight quizalofop EC), and clethodim (SelectMax, 12.6% by weight clethodim EC) were applied at two rates to the plants at the two- to three-leaf stage (Table 3.2).

Data analysis. Herbicide efficacy was estimated visually at 21 d (PRE-) or 14 d (POST-) after herbicide applications. Italian ryegrass injury was rated on a scale from 0 (no injury) to 10 (death). For PRE- herbicide studies, the experimental units with the same germination as control group were rated as 0, and the units with no emerged seedling were rated as 10. Fisher's LSD between treatments was calculated with a liner model by R software (R statistical software, R development team, http://www.r-project.org/.).

Dose-Response Study

Italian ryegrass seeds from populations R2, R4 and S were planted and treated using the same methods as described previously. R2 was selected as a representative population for populations from the small grain rotation fields (R1, R2 and R3). Flufenacet was applied at 0, 95, 190, 380, 760, 1520 and 2280 g ai ha^{-1}, while pyroxasulfone was applied at 0, 7.5, 15, 30, 60, 120 and 240 g ai ha^{-1}. The experimental design of this study was a completely randomized block with four replications (total of 56 units), and the experiment was repeated.

The percent emergence relative to the control group was determined at 21 d after herbicide application. Only seedlings that were least 1.5 cm in height were counted. Following the collection of the emergence data, seedlings were cut at the soil surface and dried at 60 C for 72 h. The percent dry weight of the treated plants relative to the control group was calculated. R and Levene's ANOVA were used to compare difference between treatments. The homogeneity of variances between populations with the same treatments was compared. GR_{50} values were estimated using the drc package (DRC package version 2.1-4) in R.

Gene Sequencing

Seedlings from R2 and R4 populations surviving the application of ACCase herbicides in the resistance screening studies were selected for gene sequencing. Fresh plant tissue was harvested independently from four healthy plants in each treatment group. Fresh plant tissue also was harvested independently from two untreated plants in the S population. Total cellular DNA was extracted immediately from harvested fresh tissue using a DNeasy Plant Mini kit (Qiagen) following the mini protocol (DNeasy plant handbook). Polymerase chain reactions (PCRs) were performed to amplify the regions of ACCase gene. Two pairs of primers were designed based on White et al. 2005 (Table 3.3). briefly, the reaction mixture contained 1.15 µl DNA solution, 0.2 µM dntp, 0.5 µM forward primer, 0.5 µM reverse primer, 1 µl 10× PCR buffer, 0.06 unit *Tag* DNA polymerase and 6.74 µl water. The PCR thermal cycler was 37 cycles each consisting of 30s at 95 C, 45s at 60 C and 2 min at 72 C, followed by a final step of 10 min at 72 C. The PCR products were purified with TOPO TA Cloning Kit for Sequencing following the manufacturer's

instructions (TOPO TA Cloning Kit for Sequencing, Invitorgen). Cloning products were purified using a QIAquick PCR Purification Kit following the vacuum protocol in QIAquick Spin Handbook. PCR products were sent to the Center for Genome Research and Biocomputing (CGRB), Oregon State University, for sequencing. Ten clones per PCR product were loaded on an automated ABI PRISM 3770 sequencer after treatment with a BigDye Terminator v.3.1 cycle sequencing kit. Each clone was sequenced in both directions. Sequencing results were edited with Finch TV and aligned using CLC Sequence Viewer v.6.1.

RESULTS

Herbicide Screening Assays

All four populations (R1, R2, R3 and R4) were resistant to flufenacet (Table 3.1). In the pyroxasulfone treatment group, all populations were controlled at application rates of 60 and 120 g ai ha^{-1}. No emerged seedlings were observed in these experimental units. Although pyroxasulfone has not yet been labeled for use in winter wheat, the 120 g ai ha^{-1} application rate is expected to be near the labeled field use rate. In the flufenacet treatment group, survivors were observed in most experimental units (Table 3.1), including the 380 g ai ha^{-1} application rate group, which is the recommended field use rate for flufenacet in winter wheat.

Screening of alternative herbicides indicated that the R2 and R4 populations have similar resistance patterns (Table 3.2). The two populations were not resistant to other Group 15 herbicides or glyphosate. However, both populations were resistant to Group 1

herbicides and mesosulfuron-methyl, a Group 2 herbicide. R4 was resistant to diuron, but R2 was not.

Dose-Response Study

There was no evidence ($P<0.05$) of differences between blocks and experiments, according to Levene's ANOVA test for homogeneity of variances. Therefore, data were pooled over the four replications and the two experiments and subjected to further analysis.

For the pyroxasulfone treatments, the S, R2 and R4 populations were controlled with no more than 10 of 50 seedlings emerging, even at the lowest application rate (7.5 g ai ha^{-1}), which is 0.0625 of the potential labeled use rate in winter wheat. Therefore, the lack of survivors prevented the determination of a GR_{50}. These results indicate that these populations of Italian ryegrass are very sensitive to low application rates of pyroxasulfone.

For flufenacet treatments, the emergence rates of S, R2 and R4 populations were negatively correlated with increasing rates of flufenacet (Figure 3.1).

Based on the regression curves of dry aboveground biomass, populations R2 and R4 were resistant to flufenacet (Figure 3.2). The GR_{50} values for R2, R4 and S were 438, 308 and 52 g ai ha^{-1}, respectively. The resistance indices (RI) for the R2 and R4 populations were 8.4 and 5.9 respectively, when compared to the S population.

Gene Sequencing

A single nucleotide change of GAT to GGT was found in the ACCase gene in the R2 population (Table 3.4). This change leads to a Asp-2078-Gly substitution. This

substitution has been reported in *Lolium spp.* previously and conferred ACCase resistance at different levels (Yu et al. 2007, Kaundun 2010).

Comparing the PCR fragments of ACCase gene from the R4 to the S populations, two alleles were found in this population. Each allele contained a different resistant gene, respectively. The first allele included an ATT to AAT mutation which leads to a Ile-2041-Asn substitution. This substitution has been reported in *Lolium rigidum* previously and conferred APPs resistance (Delye et al. 2003). The second allele included the substitution, Asp-2078-Gly, in both R2 and R4 populations.

DISCUSSION

In summary, the four Italian ryegrass populations evaluated were resistant to flufenacet but not resistant to pyroxasulfone. R2 and R4 had similar multiple resistance patterns and levels of flufenacet resistance in the dose-response study. Pyroxasulfone controlled all flufenacet resistant Italian ryegrass populations at rates greater than 7.5 g ai ha^{-1}, which is well below the proposed field rate for use in winter wheat. Both R2 and R4 populations contains a Asp-2078-Gly substitution. This substitution may contribute to the resistance to all the three ACCase classes. Another substitution Ile-2041-Asn was found in the R4 population, but previous studies indicated that this substitution only contributes to the APP resistance. Therefore, the Asp-2078-Gly substitution may still be the major reason of the ACCase cross-resistance in R4 population.

These populations are the first reported Italian ryegrass populations resistant to flufenacet in Oregon, but there was no cross-resistance to other Group 15 herbicides tested. This result indicates that either the site of action is not the same for these herbicides or that

the resistance is not site based. However, it should not be assumed that cross-resistance between these Group 15 herbicides will never occur. Further studies are needed to elucidate the mechanism of resistance and the sites of action for these herbicides.

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Table 3.1. Injury of five Italian ryegrass populations treated with flufenacet compared to the untreated control. Data of R1, R2, R3 and R4 are the average of 6 replications, while S data are the average of 24 replications.

Flufenacet (g ai ha^{-1})	Population				
	R1	R2	R3	R4	S
380	3.3a	4.0a	4.0a	3.4a	9.0b
190	2.5a	2.4a	2.8a	2.9a	8.0b

[a] Means within the same row followed by the same letter are not significantly different, according to Fisher's Protected LSD at P=0.05

b The experimental units with the same germination as the control group were rated as 0, and the units with no emerged seedlings were rated as 10.

Table 3.2. Injury of three Italian ryegrass populations treated with herbicides. Data of R2 and R4 are the average of 6 replications, while S data are the average of 12 replications.

Herbicide	Group	Timing	Rate (g ai ha^{-1})	Populations		
				S	R2	R4
metolachlor	15	PRE	2139	10.0a	10.0a	10.0a
			1069	10.0a	9.3a	9.6a
dimethenamid-*p*	15	PRE	1100	9.8a	10.0a	10.0a
			550	9.0a	8.0a	8.3a
acetochlor	15	PRE	2240	9.8a	10.0a	10.0a
			1120	9.0a	8.1b	9.1ab
diuron	7	PRE	2000	10.0a	9.3a	4.7b
			1000	8.2a	7.3a	2.7b
glyphosate	9	POST	1000[a]	9.6a	8.8a	8.6a
			500[a]	6.7a	8.3b	7.3ab
mesosulfuron-methyl	2	POST	15	9.3a	4.1b	4.5b
			7.5	7.0a	4.3b	4.6b
pinoxaden	1	POST	60	8.8a	3.8b	4.1b
			30	4.6a	2.6b	2.5b
quizalofop	1	POST	184	9.6a	4.5b	3.5b
			92	6.6a	4.2ab	2.5b
clethodim	1	POST	136	9.1a	5.5b	5.0b
			68	6.7a	3.8b	3.3b

[a] g a.e. ha^{-1}

[b] Means within the same row followed by the same letter are not significantly different, according to Fisher's Protected LSD at P=0.05

[c]Plant injury value 0 = no injury observed, 10 = no emergence (PRE) or dead (POST)

Table 3.3 Primers used for sequencing ACCase gene from R2 and R4.

Primer	Sequence
CP1-F[a]:	5'-CAAACTCTGGTGCTCGGATTGGCA-3'
CP1-R:	5'-GAACATAGCTGAGCCACCTCAATATATT-3'
CP4-F:	5'-CAGCCTGATTCCCATGAGCGGTC-3'
CP4-R:	5'-CCATGCATTCTTGGAGTTCCTCTGA-3'

[a] F: forward; R: Reverse

Table 3.4 Comparison of ALS gene sequences from R2, R4 and S populations

Amino acid	G2040	I2041	L2042	~	I2077	D2078	S2079
Sequence	GGA	ATT	CTG	~	ATT	GAT	AGC
S	-[a]	ATT	-	~	-	GAT	-
R4-1	-	AAT\N	-	~	-	GAT	-
R4-2	-	ATT	-	~	-	GGT\G	-
R2	-	ATT	-	~	-	GGT\G	-

[a] –there was no difference of protein coding nucleotides between the two populations.

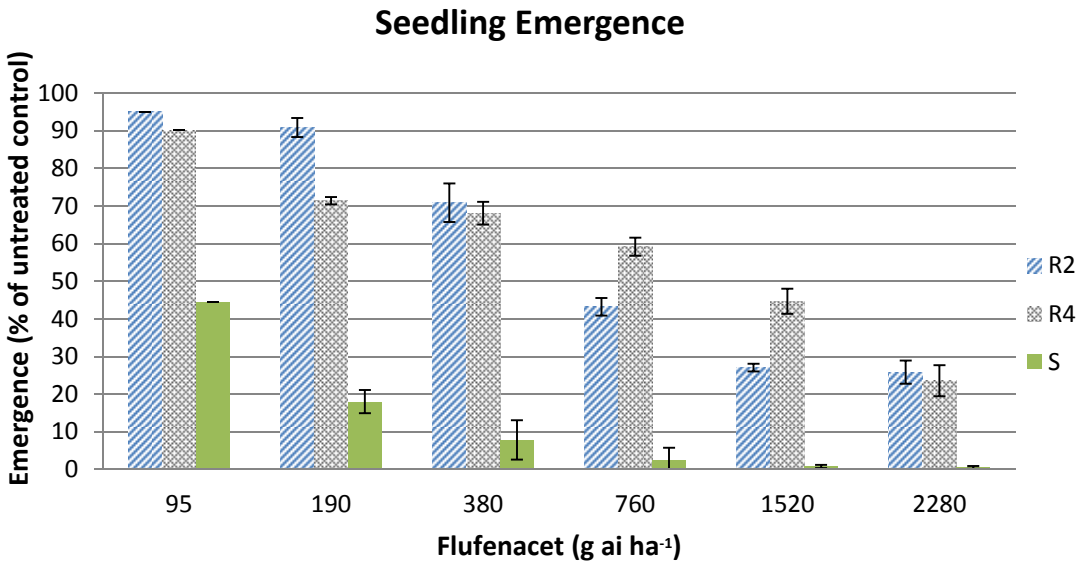

Figure 3.1. Seedling emergence of three Italian ryegrass biotypes treated with flufenacet. Data for R2 and R4 were pooled over 8 replications and data for S over 16 replications of the same dose (with standard error).
$LSD_{0.05}$ between R2 and R4 = 18.9%
$LSD_{0.05}$ between R2, R4 and S = 16.4%

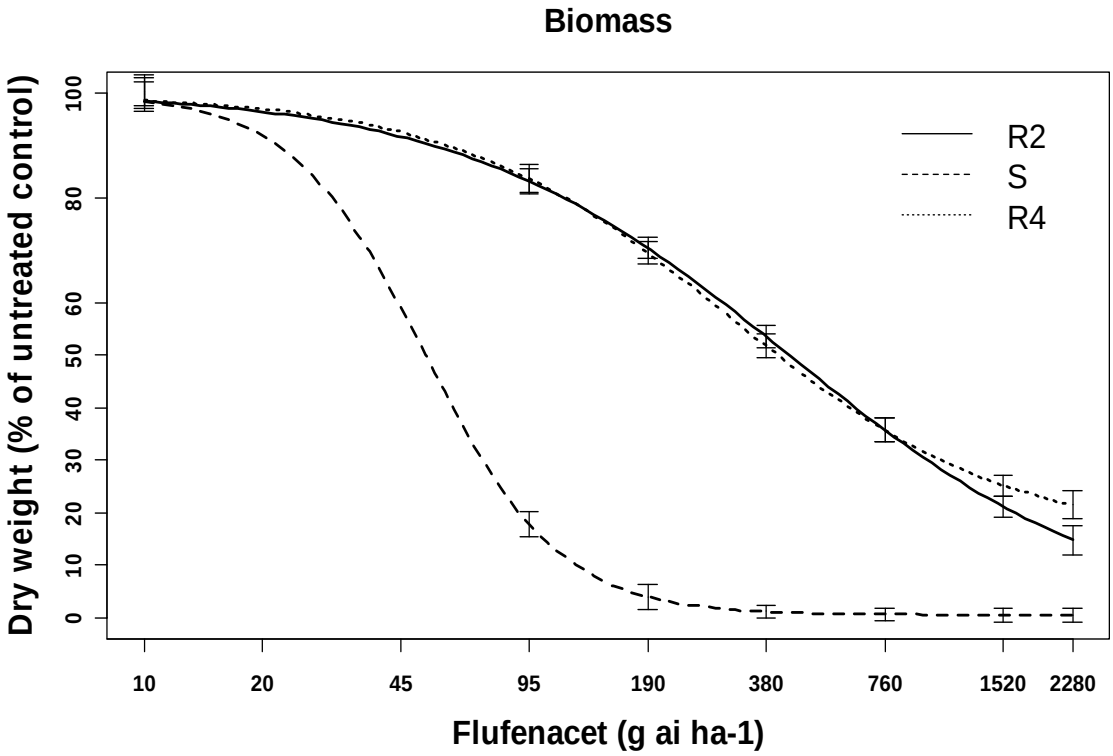

Figure 3.2. Aboveground biomass dry weight as % of untreated control of three biotypes treated with flufenacet. Data for R2 and R4 were pooled over 8 replications and data for S over 16 replications of the same dose. Each point is the mean ± SE. The lines are log-logistic curves: R2:$Y=-0.64+101.06/(1+e^{[1.04\times\log(x)+0.37]})$; R4:$Y=14.85+84.94/(1+e^{[1.24\times\log(x)+0.63]})$; S:$Y=0.49+99.51/(1+e^{[2.55\times\log(x)+3.28]})$.

CHAPTER4: SUMMARY

Five Italian ryegrass populations were screened for herbicide resistance as part of this thesis research project. All five populations were confirmed to be resistant to at least one herbicide. Three of the populations were studied more intensely to quantify their resistant levels and identify the resistance mechanisms. Multiple-resistance was identified in these three Italian ryegrass populations.

The CT population was resistant to four herbicide groups, Group 2, 5, 7 and 9. The R1, R2, R3 and R4 populations were resistant to flufenacet. The R2 and R4 populations were selected for further study and had similar multiple-resistant patterns. Both R2 and R4 were resistant to flufenacet and cross-resistant to several ACCase herbicides.

Although not all the resistance mechanisms were identified in these Italian ryegrass populations, several target-site based resistance mechanisms were identified. Two substitutions, Trp591 to Leu in the ALS gene and Ser264 to Gly in the *psb*A gene, were found in the CT population, and contribute to ALS and PSII resistance, respectively. Two other substitutions, Asp-2078-Gly, found in R2 and R4 populations, and Ile-2041-Asn found in R4 population contribute to ACCase resistance. All of the substitutions have been reported previously (Beckie and Tardif 2012).

Though cultural and mechanical controls should be considered in herbicide resistance management programs, herbicides are still the first choice of weed control in commercial crop production systems. Herbicide and crop rotation are two basic methods for resistance management in herbicide based weed control systems.

In this study, two groups of Italian ryegrass populations from two different cropping systems in the same area (Mid-Willamette Valley, Oregon), without barriers for gene flow were identified. It is possible that gene flow could move resistance genes from these populations to other ryegrass populations. Selection of several different resistant biotypes in one agricultural area makes herbicide rotation more difficult. Simply changing to a herbicide with different MOA may not effectively control these multiple resistant populations. However, a new herbicide, such as pyroxasulfone, could be used to control these multiple resistant populations, but over dependence could result in resistance to it over time.

For better resistance management, several further studies need to be done. The first one is to determine the progeny fitness of the resistant biotypes. As reviewed previously, Italian ryegrass influences the cropping system by competing with the crop for resources, and additionally is widely spread because of high seed production. Information on resistant biotype fitness, such as seed germination, growth rate, seed production, and competitiveness could be helpful in developing resistance management strategies. Determination of fitness levels may help to determine the economic threshold for management. Additionally weed surveys need to be conducted to investigate the resistance patterns, even in unselected populations, in this area. These surveys may identify how resistance developed and remains in the cropping systems and may be useful to predict resistance in the future.

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